An Innovative Method for Measuring Cortical Acetylcholine Release in Animals on a Second-By-Second Time Scale

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by

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ABSTRACT

Acetylcholine (ACh) is critical for normal attentional processing and is implicated in the craving and relapse cycle of addiction. Microelectrode technology provides a novel second-by-second way of measuring the effects of drug administration on neurotransmitter release and degradation, and vastly improves upon spatial and temporal resolution of microdialysis. Ejections of nicotine (1 mM, 80 nL), an addictive drug that has been shown to increase cortical ACh release, were hypothesized to result in ACh signal peaks that rapidly attained peak amplitudes and then were rapidly cleared to baseline. It was also hypothesized that because the ACh signal is dependent upon the degradation of ACh by acetylcholinesterase (AChE), the administration neostigmine (10 μM), an AChE inhibitor, would markedly attenuate nicotine-evoked ACh signal peaks. Nicotine and neostigmine were administered in the prefrontal cortex in seven anesthetized rats. After baselines were collected, nicotine was ejected and evoked ACh release was measured for peak amplitude and clearance time (average pre-neostigmine amplitude was 3.58 μM and average pre-neostigmine clearance time (t80) was 6 sec). Following the nicotine ejections, neostigmine was perfused through a dialysis probe. Nicotine ejections were repeated. Following neostigmine exposure, the ACh signal peaks and clearance times were markedly attenuated (average post-neostigmine amplitudes decreased 71% to 0.89 μM and average t80 increased to 8 sec). Clearance rates (μM/sec) were also attenuated by 85% after neostigmine administration. Microdialysis collections were taken for the duration of the experiment and analyzed for ACh concentrations. Time controls and saline ejection controls were also performed as negative controls.

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The systems mediating cortical acetylcholine (ACh) release are critical in normal attentional processing (Sarter & Bruno, 1997) and dysfunctions in these systems are postulated to underlie the phenomena of craving and relapse associated with drug addiction (Hyman, 2005; Nestler, 2002). A better understanding of the factors regulating ACh release would lead to a greater knowledge of the neural circuits underlying drug addiction, as well as facilitate the development of more effective pharmacotherapies. Our novel ACh microelectrode arrays, with their superior spatial and temporal resolution and self referencing ability, will greatly contribute to this goal by allowing the measurement of ACh release on a second-by-second basis. The validation and subsequent use of ACh-sensitive microelectrode arrays will create a paradigm shift in studies on the relationship between cholinergic transmission and behavior.

Acetylcholine, Attention, and Addiction

The basal forebrain cortical cholinergic system (BFCS) is a major source of the mediation of cortical ACh release. The basal forebrain, specifically the areas of the nucleus basalis (the anteromedial and intermediate areas) and substantia innominata (Wenk, 1997; Zaborszky et al., 1999; Mesulam & Geula, 1988), projects cholinergic afferents to the cerebral cortex. Cholinergic axons have been found in all cortical areas and cell layers of the cortex, in varying densities (Mesulam, 1992; Eckenstein et al., 1988). Initially, it was hypothesized that despite the varying densities of cholinergic axons, the efferents from the basal forebrain acted in a global, homogenized fashion. However, as more data has been collected, it is apparent that

different brain areas respond differently to stimuli that effect basal forebrain neurons (Sarter et al., 2005a). ACh release in the cortex is thought to modulate the ability of other neurons in the brain to select and process behaviorally important stimuli (Sarter et al., 2001). Sensory stimuli (e.g. auditory and visual stimuli) are a major factor contributing to the release of ACh (Bakin & Weinberger, 1996), as the BFCS inputs are implicated in mediating the ACh release that is correlated with sensory stimulation (Gill et al., 2000) and lesions of the basal forebrain result in less visual cortex neuronal activity when exposed to visual stimuli (Sato et al., 1987). The BFCS and ACh release play a role in gating relevant sensory stimuli (Sarter et al., 2001). Gating is important in the detection and selection of relevant stimuli, and therefore, attentional processes. However, there are still facets of attentional processes that need clarification. The use of our novel microelectrode array will allow for the exploration of *in situ* activity, thus providing a better picture of the relationships between functionally distinct brain areas, ACh release in response to stimuli, and attention.

To demonstrate that cortical cholinergic transmission is necessary for performance in attentional tasks, studies using the selective immunotoxin 192 IgG-saporin were performed. McGaughy et al. (1996) utilized 192 IgG-saporin-induced lesions in the nucleus basalis/substantia innominata area of the basal forebrain in rats previously trained in a sustained attention task. After lesioning, the rats' ability to detect signals during the task was diminished, however correct rejections remained stable (providing more evidence for the role of the BFCS in stimulus processing). Additionally, in lesioned rats, cortical AChE positive fibers were reduced, and this reduction correlated with a decrease in performance. Even in rats with relatively moderate losses of BFCS projections (induced through multiple small applications of 192 IgG-

saporin), impairments in a sustained attention task were apparent (McGaughy & Sarter, 1998). Animals trained to pair a food stimulus with the onset of darkness showed an increase in ACh efflux when presented with the stimulus, but ACh efflux was reduced in lesioned animals (Fadel et al., 1996), and histology showed a decrease in the number of AChE positive fibers in these animals. The administration of a nicotinic ACh receptor agonist in basal forebrain-lesioned rats did not reverse the effects of lesioning on task performance (McGaughy et al., 1999). Together, these findings indicate that a loss of corticopetal projections from the basal forebrain result in attentional deficits.

Further studies showed that performance in an attentional task is sufficient to increase ACh release. Microdialysis studies measuring ACh efflux in the frontal cortex found that rats exposed to novel stimuli experience significant increases in frontal ACh release, while habituated rats do not show significant increases (Acquas et al., 1996). The significant increases in ACh efflux associated with novel stimuli were hypothesized to reflect the activation of BFCS projections in the cortex, as well as arousal and attention associated with novel stimuli. Passetti et al. (2000) found that in rats performing a sustained attention task, there were robust increases in ACh release in the medial prefrontal cortex. However, the level of attentional difficulty of the task did not have an effect on the amount of ACh efflux. A study by Himmelheber et al. (2000) measured ACh efflux in the frontoparietal cortex of rats during a sustained attention task. Himmelheber and colleagues found that ACh efflux increased upon placing the rats in their operant chambers, thus suggesting arousal or conditioning. ACh efflux further increased with the onset of the task, and the presence of a distractor (and thus an increased attentional demand) resulted in an additional increase in ACh efflux. In contrast to Passetti et al.'s findings, these

data suggest a relationship between ACh efflux in the cortex and level of attentional demand during a task. Additionally, the sustained attention task has been shown to produce far more marked increases in ACh efflux than operant control tasks that require little attention (Arnold et al., 2002).

The BFCS has been shown to be both necessary and sufficient for the increases in ACh release associated with normal attentional processing. Several neuropathologies, including Alzheimer's Disease (Wenk & Willard, 1998), schizophrenia (Sarter et al., 2005b), and drug addiction (Sarter & Bruno, 1999) are hypothesized to be the result of malfunctions within the BFCS and associated neural circuitry (Sarter & Bruno, 1997). A large number of studies have investigated the disregulation of BFCS through microdialysis methods, and vast amounts of information have been gained. However, to fine tune this information and understand more exactly the causes of these neuropathologies, methods with better spatial and temporal resolution are essential.

Traditional theories of drug addiction center around the reward associated with DA release and regulation in the ventral tegmental area (VTA), NAC, amygdala, prefrontal cortex (PFC), and other forebrain areas (Kelley & Berridge, 2002; Hyman, 2005; Di Chiara, 1999). The BFCS is an integral part of the neural circuits involved in drug addiction, as the basal forebrain (and consequently cortical ACh release) is influenced through dopaminergic input from the VTA (Gaykema & Zaborszky, 1996), GABAergic input from the NAC (Zahm & Heimer, 1993; Zaborszky & Cullinan, 1992) and glutamatergic input from the PFC (Zaborszky et al., 1997) (see Figure 1 from Sarter et al., 2005b). Activation of DA neurons in the VTA has been shown to be

correlated with learning about cues that predict reward in monkeys (Hollerman & Schultz, 1998). Thus, it can be hypothesized that the basal forebrain receives excitatory input from the VTA that reflects learning reward predictability (Hyman & Malenka, 2001). Similarly, the NAC also receives dopaminergic projections from the VTA, as well as glutamatergic input from the prefrontal cortex (Kalivas & Volkow, 2005). To discern the role of the NAC in influencing the BFCS, many pharmacological manipulation studies have been performed. In one study, injections of DA into the NAC, as well as injections of D_1 followed by D_2 agonists, resulted in increases in neuronal activity in the ventral pallidum (Yang & Mogenson, 1989). In another study, the infusion of a D_1 receptor antagonist into the NAC slightly attenuated cortical ACh efflux, while the infusion of a D_2 receptor antagonist into the NAC markedly attenuated ACh efflux (Moore et al., 1999; Sarter et al., 1999). In rats performing a task, however, it was found that a D_2 antagonist perfused into the NAC did not attenuate increases in ACh release (Neigh et al., 2004), and the study concluded that neuronal activity in the NAC is necessary for increases in ACh associated with the demands of the task. Finally, infusion of the D_2 antagonist raclopride increased extracellular levels of GABA in basal forebrain regions (Ferre et al., 1994). All of this evidence taken together suggests that dopaminergic activity in the NAC disinhibits the GABAergic projection to the basal forebrain and allows DA activity in the NAC to modulate cortical ACh release. The glutamatergic projection from the PFC (particularly the anterior cingulate and orbitofrontal regions) to the NAC also contributes to the modulation of ACh release (Kalivas & Volkow, 2005). The third projection to the basal forebrain mentioned previously, the glutamatergic projection from the PFC, also plays a role in modulating cortical ACh release. Basal forebrain glutamate receptor stimulation contributes to ACh efflux in response to a stimulus paired with a reward (Fadel et al., 2001).

From the evidence presented, it is clear that both the BFCS and the mesolimbic dopaminergic system are complicated systems that influence ACh efflux. The role of these systems in drug addiction is equally complex. Repeated psychostimulant drug use (i.e. highly addictive drugs such as cocaine or amphetamine) has been found to change branching patterns of neurons in rats (Robinson & Kolb, 1999). Repeated use of these drugs resulted in an increased number of dendritic branches and increased dendritic density in both the NAC and PFC. Amphetamine use has also been shown to affect a number of factors, including ACh release and attention. Systemic amphetamine administration increases basal cortical ACh release, as well as NAC DA efflux (Arnold et al., 2000). This study also utilized D_1 and D_2 receptor antagonists in the NAC to explore the relationship between NAC DA activity and amphetamine-induced ACh release. It was concluded that because the antagonists had little effect, the systems mediating systemic amphetamine-induced ACh release were widespread and utilized multiple signaling pathways. However, a subsequent study found that ACh release due to amphetamine administration can be attenuated by inhibition of the cholinergic projections within the basal forebrain (Arnold et al., 2001). Pretreatment with amphetamine has been shown to increase cortical ACh release when the drug is administered days later (Nelson et al., 2000) and repeated administration of amphetamine has been found to produce attentional impairments in a sustained attention task (Deller & Sarter, 1998), with sensitized rats showing increased false alarm response rates. The sensitization of cortical ACh release by amphetamine administration serves as evidence for the hypothesis that the problems of craving and relapse in drug addiction are due to drug-induced ACh sensitization and thus overprocessing of drug related stimuli. In humans, the possibility of overprocessing of drug related stimuli can be seen in a Stroop task testing for bias in cocaine users (Hester et al., 2006). The cocaine users showed a strong attentional

preference for cocaine related pictures and words. Alcoholics also exhibit a preoccupation with alcohol related stimuli, and report increases in craving when presented with such stimuli (Ingjaldsson et al., 2003). Using PET scans, researchers have also been able to link increased craving with hyperactivity in the cortex (Volkow et al., 2002). All of these findings point to a relationship amongst attention, acetylcholine, and addiction. This study aims to collect additional data supporting this relationship through the use of a novel method.

This current study focuses on nicotine, a highly addictive drug, and its role as a potent stimulator of ACh release in the medial PFC (mPFC). Similar to amphetamine, repeated administration of nicotine results in sensitized increases in ACh release in the cortex (Arnold et al., 2003). Nicotine and the increases in cortical ACh release associated with it may be the reason for improved performance in attentional tasks after nicotine administration (Stolerman et al., 2000). Nicotine has also been shown to increase DA release in the NAC, another area heavily implicated in addiction and reward (Balfour et al, 2000). In humans, smokers have been found to attend to smoking related stimuli longer and more intensely when craving levels are high (Mogg et al., 2005; Waters et al., 2003). Nicotine is an ideal drug to use in this study because it satisfies the criteria of affecting cortical ACh release, being an addictive substance, and having an effect on attention.

Microelectrode Array

There are many ways of studying extracellular ACh in the brain. A goal of this research is to provide further validation of a novel technique, the microelectrode array. Until recently, the standard method of obtaining data regarding neurotransmitter efflux was through the use of

microdialysis. However, microdialysis is limited by its temporal and spatial resolution. Microdialysis collections at best can be taken every 5-8 min, thus allowing researchers to only see the summation of thousands of neurochemical events. While able to provide a tonic view of neurotransmitter activity (which is an important component of neurotransmitter release), microdialysis is unable to provide a phasic view of this activity. The ability to measure the phasic component of neurotransmitter activity is important for being able to link transient neurotransmitter changes with rapidly occurring changes in behavior. Also, with a harvesting radius of approximately 500-1000 μm, microdialysis cannot be used to discern functionally distinct areas of the brain (Bruno et al., 2006).

As an alternative to microdialysis methods for measuring neurotransmitter activity, a variety of microsensors were developed. These microsensors (or microelectrodes) have been used to measure in vivo levels of neurotransmitters (Wilson & Gifford, 2005). Carbon fiber microcylinder electrodes have been used with horseradish peroxidase and choline oxidase (ChO) for the measurement of choline (Ch) (Garguilo & Michael, 1996), and platinum iridium wire electrodes have been used for measuring both ACh and Ch (Mitchell, 2004). These microelectrodes improved upon the spatial and temporal resolution of microdialysis methods. The response rates for these microelectrodes ranged in seconds, as opposed to the minutes of microdialysis collection intervals. Additionally, many microelectrodes were smaller in outer diameter size than microdialysis probes (5-30 μm for microelectrodes versus 150-500 μm for microdialysis membranes), thus causing less damage to the brain areas being investigated (Bruno et al., 2006). However, these microsensor techniques lack the sophisticated self referencing ability of the microelectrode array, as the microsensors can only generate a single signal from the microsensor itself.

The microelectrode array is a novel technology that drastically improves upon the spatial and temporal resolution deficits of microdialysis methods and the lack of self referencing of other traditional microelectrodes. The microelectrode array contains four platinum sites, each 15 x 333 μm, which allow for *in situ* recordings of neurotransmitter activity. Additionally, the microelectrode array is capable of up to ten readings per second, a dramatic improvement over the microdialysis method (Burmeister et al., 2000), as well as some microelectrodes. The availability of four separate recording sites on the microelectrode array allows for the ability to subtract noise and other interferents from the generated signals, thus leaving a truly self referenced signal. The microelectrode array technology will allow for a better understanding of the dynamics of neurotransmitter activity, and in turn, a way to measure rapid processes such as the release of ACh in response to an addictive drug (Parikh et al., 2004).

To explore the relationship between an addictive drug and ACh release, as well as to validate our microelectrode array, two hypotheses for this study were developed. It was hypothesized that ejections of nicotine in the PFC of anesthetized rats, implanted with microelectrode arrays, would evoke ACh signal peaks that would rapidly attain peak amplitude, and then be rapidly cleared to baseline.

The second hypothesis of this experiment is that the signal generated by the microelectrode array is dependent upon the conversion of ACh by AChE. This hypothesis will be manipulated through the use of neostigmine, an AChE inhibitor. The microelectrode array requires that ACh be broken down by AChE in order for electrons to be eventually donated to the platinum recording site. By locally inhibiting endogenous AChE activity in the brain using

neostigmine, it is expected that the microelectrode array ACh signal would be significantly attenuated.

METHOD

Subjects

Seven adult male Wistar rats weighing between 250 and 400 g were obtained from Harlan Laboratories (Indianapolis, IN, USA). Animals were individually housed in a temperature and humidity controlled room kept on a 12:12 light:dark cycle (lights on at 6:00 am) with ad libitum access to food and water. Animals were treated in accordance with the guidelines of the Institutional Laboratory Animal Care and Use Committee (ILACUC) at The Ohio State University and animal care and experiments were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Drugs and Chemicals

ChO, AChE, bovine serum albumin (BSA), glutaraldehyde, ascorbic acid (AA), dopamine (DA), norepinephrine (NE), Ch, ACh, nicotine, neostigmine, and m-Phenylenediamine dihydrochloride (m-PD) were all purchased from Sigma (Sigma Chemical, St. Louis, MO, USA). Ultra pure water (RO-HPLC grade, Millipore, Billerica, MA, USA) was used to prepare all solutions. Solutions for intracranial administration were prepared in 0.05M phosphate-buffered saline (PBS) and pH was adjusted to 7.2-7.4.

Preparation of Microelectrode Array

Ceramic-based microelectrodes were obtained from Thin Film Technology (Buellton, CA, USA) and Hybrid Circuits, Inc. (Sunnyvale, CA, USA). The S-2 type microelectrode has two pairs of platinum recording sites. Each site measures 15 x 333 μm, and the sites begin approximately 100 μm from the tip of the microelectrode (see Figure 2). Upon arrival, the microelectrode was coated with a thin layer of epoxy to prevent contamination of the channels on the paddle. The microelectrode tip was then cleaned with Citrisolve (Fisher Scientific), isopropyl alcohol, and ultra pure water. After drying, the Ch sensitive top pair of sites was coated with a solution of 1.2% ChO, 1 % BSA, and 0.12% glutaraldehyde in water. The bottom pair of sites, sensitive to both ACh and Ch, was coated with a solution identical to the one above, except with the addition of 0.07% AChE. A 1.0 μl syringe was used to apply the coatings (Hamilton Co., Reno, NV, USA). Four to five coatings were applied, and the microelectrode was then cured for 12 hours at 42° C. After curing, the microelectrode was allowed to cool to room temperature before use.

A micropipette puller was used to obtain micropipettes with the internal diameter of 10-15 μm. The micropipette was affixed to the microelectrode array platform using sticky wax. The spacing between the tips of the micropipette and ACh/Ch microelectrode was 80-100 μm. On the day of the experiment, the microelectrode was electropolymerized (0.5 V) in 0.05 M PBS containing m-PD (5.0 mM) for one hour to apply a coat sufficient to block electroactive interferents such as AA, DA, and NE.

In Vivo Recordings

The ACh specific microelectrode array consists of differential enzyme coatings (see Figure 3). The AChE layer allows for the hydrolysis of ACh into Ch. The Ch is then subsequently oxidized into H_2O_2 by ChO. The molecules of peroxide are small enough to fit through the exclusionary m-PD layer. The H_2O_2 molecules donate electrons to the platinum sites, which generate a current. Within the software, the current is then converted from nA to μM concentration.

The first pair of sites closest to the tip of the microelectrode array is coated in AChE and ChO. The second pair of sites is coated in only ChO. The signal from the second pair of sites is subtracted from the signal from the first pair of sites, leaving only the self-referenced ACh signal. The subtraction of the Ch signal from the combined ACh/Ch signal also allows for the elimination of background noise in the signal, assuming the noise would be reported on both pairs of sites (Burmeister & Gerhardt, 2001).

In Vitro Calibration of Microelectrode Array

On the day of the experiment, the microelectrode array was calibrated using the FAST-16 recording program (Quanteon, LLC, Lexington, KY, USA). Constant voltage amperometry was used with a potential of +0.7 V applied versus an Ag/AgCl reference electrode. Calibration was performed in a stirred solution of 0.05M PBS (40 ml) at 37ºC. The microelectrode was allowed to stabilize, and then aliquots of stock solutions of AA (20 mM), ACh (20 mM), and Ch (20 mM) as well as DA (20 mM) and NE (2 mM) were added to the beaker such that the final concentrations were 250 μM AA, 10, 20, 30, and 40 μM ACh, 20 μM Ch, 20 μM DA, and 2 μM

NE (see Figure 4). The slope, limit of detection (L.O.D., e.g. minimum μM with signal:noise of 3:1) and linearity (R^2) for ACh and Ch, as well as selectivity ratio for AA, DA, and NE were calculated (Bruno et al., 2006). The Ch slope similarities for the MEAs fell in the range of 80% - 125%.

Microdialysis

Microdialysis probes (SciPro, Inc., 0.15 mm, o.d., 3 mm membrane tips) were used to take dialysate collections. Collections were taken every 15 minutes. Probes were perfused with artificial cerebral spinal fluid (aCSF: pH = 7.0, 155 mM Na^+, 2.9 mM K^+, 1.1 mM Ca^{+2}, 0.83 Mg^{+2}, 132.8 mM Cl^-, and 5.9 mM glucose) unless otherwise specified.

Analysis of ACh Dialysate

High Performance Liquid Chromatography (HPLC) coupled with electrochemical detection was used to analyze levels of ACh in dialysate collections. The HPLC analysis allowed for detection of ACh concentrations as low as 1.0 fM per 15 mL. Samples were stored at -80º C until analyzed. For the detailed procedure, see Zmarowski et al. (2005).

Procedure

On the day of the experiment, the animals were anesthetized with urethane (1.25 g/kg, i.p.). The rats were then placed in a sterotaxic frame and a heating pad at 37ºC maintained the rats' body temperature. A miniature (200 μm in diameter) Ag/AgCl reference electrode was implanted in the parietal cortex (AP:-4.0 mm, ML:-2.0 mm, DV:-1.0 mm, measured from bregma), contralateral to the MEA and dialysis probe.

A microdialysis probe was positioned within the mPFC, without a guide cannulae. The probe was positioned posterior, relative to the microelectrode recording site, with the top of the probe angled anterior at 30º (AP:5.6 mm, ML:-0.55 mm, DV:-4.9 mm, measured from bregma). This positioned the probe tip approximately 500 µm from microelectrode recording sites. The probe was perfused with aCSF at a rate of 1.25 µl/min. The probes were attached to a dual channel liquid swivel (Instech, Plymouth Meeting, PA, USA).

Two hours after probe insertion, the microelectrode/micropipette assembly was inserted vertically into the mPFC (AP: +2.7 mm, ML: -0.7 mm, DV: -4.2 mm, measured from bregma). The brain was allowed two hours to equilibrate before nicotine ejections began.

Nicotine was ejected (over a period of 0.1 sec) from the micropipettes using a pressure ejections system (Picospritzer II, Parker Hannifin Co., Fairfield, NJ). The resulting ACh/Ch or Ch sentinel microelectrode signals were recorded from the four microelectrode recording sites using the FAST-16 recording system. Four ejections of nicotine (with 2 minutes separating each ejection) were administered. After allowing the microelectrode signals to return to basal levels following the fourth nicotine ejection, the aCSF perfusion was switched to neostigmine. Neostigmine was allowed to perfuse for 90 minutes in order to thoroughly saturate the region around the MEA tip. After 90 minutes, the four nicotine ejections were repeated. In the time control group, aCSF was perfused during the entire duration of the experiment.

Histology

At the end of the experiment, the rats were transcardially perfused with 0.2% heparin in 0.9% saline followed by 10% formalin. The brains were stored in 10% formalin for at least 24 hours at 4ºC, and then transferred to a 30% sucrose solution for at least 72 hours. Sagittal brain sections (50 μM thick) were sliced and stained using cresyl-violet to check the placement of the microdialysis probe and MEA.

Design and Statistical Analysis

The experimental design is a repeated measures design. The seven rats were each given a series of nicotine ejections (1 mM, 80 nL) and then the nicotine series was repeated after the PFC was perfused with neostigmine. Not included in the above design are a saline control and a time control. The saline control received ejections of saline to ensure that the nicotine-induced ACh peaks were not merely due to the pressure ejection of liquid into the brain. The purpose of the time control was to ensure that any attenuation of the ACh signal was not due to time or nicotinic receptor desensitization. The time control received ejections of nicotine, but was perfused with aCSF throughout the experiment. The nicotine ejections (data taken in terms of pre- and post-neostigmine administration), served as a repeated measures independent variable. The dependent variables measured were peak amplitude (nA, or expressed as μM equivalent), clearance rate, and t80 (amount of time for 80% of the signal to clear). Microdialysis collections were also taken during the course of the experiment.

RESULTS

Histological Analyses

Examination of the histology led to the exclusion of two data sets, due to the amount of damage done to the mPFC. The histology for the remaining five subjects indicated that the MEAs and dialysis probes were correctly placed within the mPFC. A representative sagittal section is seen in Figure 5.

Effects of Nicotine on ACh Signal

The analysis of the series of four pre-neostigmine nicotine-induced ACh peaks showed no significant difference amongst the peaks (see Figure 6). Therefore, the peaks were shown to be reliable through the use of a repeated measure ANOVA (GROUP, $F_{3,12} = 0.701$, $p > 0.05$).

The ACh signals quickly attained their peak amplitudes following nicotine ejections before the administration of neostigmine. The rise times ranged from 1.0-2.0 sec to reach the maximum amplitudes of the peaks.

In order to simplify the statistical analysis, analyses were focused on the third ejection of each series. As the peaks were found to be reliable, the use of the third ejection for data analyses is plausible.

Dependent variable group data were collected in terms of pre- and post- neostigmine administration (Figure 7). The data show that rapidly attained peak amplitudes were quickly cleared to baseline. The average peak amplitude was 3.58 μM, and the average t80 was 6 sec.

The average clearance rate was 1.61 μM/sec. It is interesting to note that the pre-neostigmine amplitude values ranged from 0.77-10.3 μM. This variability will be addressed in the discussion section.

Saline Control

A paired samples t test was performed to ensure that simply the procedure of ejecting a solution into the brain did not produce the signals seen in Figure 6. The peaks resulting from saline ejections were significantly smaller than the nicotine-induced peaks (see Figure 8). Analysis found that there was a significant difference between saline-induced and nicotine-induced peak amplitudes ($t(3) = -19.497$; $p < 0.05$).

Effects of Neostigmine on ACh Efflux

Neostigmine was found to be quite effective in inhibiting AChE as evidenced by the dramatic increases in ACh efflux. Figure 9 demonstrates the marked increase in percent change from ACh baseline following the administration of neostigmine. Microdialysis collections were analyzed and the increase in ACh concentration was found to be significant (DIALYSATE, $F_{1,4} = 10.244$, $p < 0.05$).

Effects of Neostigmine on Nicotine-Induced ACh Signals

The 90 minute perfusion of neostigmine attenuated the rise times to peak amplitude (Figure 6). The range of rise times increased to 8-10 sec.

The administration of neostigmine significantly attenuated the amplitude and clearance rates of nicotine-induced ACh signal peaks (Figure 6). The average peak amplitude dropped to 0.89 μM (a decrease of 71%), and the average clearance rate dropped to 0.25 μM/sec (Figure 7).

Due to the large variability of the data, the data were analyzed using a nonparametric Wilcoxon Rank Sum Test for the comparison of means. The amplitude attenuation of the ACh signal by neostigmine was found to be significant ($z = 0$, $p < 0.05$). The attenuation of the clearance rate by neostigmine was also found to be significant ($z = 0$, $p < 0.05$).

Although there appeared to be a slight attenuation of the t80 times due to neostigmine, an analysis could not be performed. The t80 times could not be analyzed because there were not enough subjects after one data set was excluded for not having a difference between pre- and post-neostigmine times.

Time Control

To ensure that peak amplitude attenuation after the administration of neostigmine was not simply due to the 90 minute delay coupled with nicotinic receptor desensitization, a repeated measures ANOVA was performed. Two groups of four nicotine ejections, separated by a 90 minute perfusion of aCSF instead of neostigmine, were analyzed against each other (Figure 10). There were no significant differences between the first set of ejections and the second set of ejections 90 minutes later (TIME, $F_{1,3} = 0.809$, $p > 0.05$).

DISCUSSION

The goals of this study were (1) to demonstrate that ejections of nicotine into the mPFC evoke ACh release with a temporal resolution that far exceeded that of conventional microdialysis and (2) to show the necessity of endogenous AChE for generating the signal of an ACh-sensitive MEA. The data collected provide support for these goals. The interpretation of the results and the general implications of these findings will be discussed in this section.

Interpretation of Nicotine-Induced ACh Signal

The results of this study support the first hypothesis. It was hypothesized that ejections of nicotine, a highly addictive drug that has previously been shown to increase cortical ACh release (Arnold et al., 2003), would result in peaks that rapidly attain peak amplitude and then are rapidly cleared. The ejections of nicotine did result in increases of cortical ACh release, as evidenced by the ACh signal peaks (e.g. Figure 6). With average rise times of 1.0-2.0 sec to reach peak amplitude following nicotine administration, it is obvious that the ACh release in response to nicotine ejections is an extremely rapid process. Clearance times averaged 6.0 sec. While the clearance times were slower than the rise times, the clearance times also showed themselves to be rapid. It would be expected that the clearance times would be longer than the rise times, as ACh release in response to nicotine probably occurred over a few seconds (the time it took the nicotine to diffuse from the pressure ejection site and stimulate additional ACh release).

The established relationship between nicotine and ACh release was supported by our findings. Nicotine is a nicotinic receptor agonist, thus stimulation of the receptors by nicotine

causes a release of ACh. The variability in peak amplitude μM concentrations (ranging from 0.77 μM to 10.30 μM) can be attributed to several factors. First and foremost, the variability may be due to individual differences amongst the animals used. A number of genetic and environmental factors may have influenced each animal's ability to respond to nicotine with ACh release. Thus, some animals may respond less to nicotinic receptor stimulation (0.77 μM peaks) or more strongly to nicotinic receptor stimulation (10.30 μM peaks). ACh response to repeated nicotine administration has been found to be relative to the animals' initial responses to nicotine (Arnold et al., 2003), thus providing evidence for individual differences in ACh response to nicotine. Additionally, the range of peak amplitudes may reflect the different basal levels of ACh within the animals' brains. The variability may also be due to individual microelectrode array sensitivity to ACh. Although each microelectrode array used fell within our parameters, it is possible that the combination of an animal's response to nicotine, as well as its basal levels, and the range of our parameters produced divergent peak amplitudes.

While the behavioral effects of nicotine could not be explored in this study due to the anesthesia, the release of cortical ACh release in response to an addictive drug is evident. As the BFCS regulates cortical ACh release, and the basal forebrain itself is influenced by the cortex, VTA, and NAC, the effects of nicotine-induced ACh release produce widespread effects in the brain that may lead to the attentional effects associated with nicotine administration (Stolerman et al., 2000).

Saline Control Interpretation

The saline ejection control data served to eliminate the possibility that the nicotine-induced ACh release was simply due to the ejection of a solution into the brain. The ACh released in response to neural trauma near the tip of the micropipette from the pressure ejections was negligible. This differs dramatically from the area affected by microdialysis. As microdialysis probes harvest from a radius of 500-1000 μm, it is necessary that the vehicle (aCSF) be perfused throughout this area. Histology of brains used for microdialysis show a visible collection radius around the tip of the probe, thus indicated neural damage within this radius. Additionally, microdialysis probes cause more damage than the microelectrode arrays, due to their larger size (see Figure 5).

The analysis of the saline ejection control showed that the peaks (or lack there of) were significantly smaller than the nicotine-induced ACh peaks. The slight negative deflections in the ACh signal that appear in Figure 8 are due to a "washout" of ACh near the tip of the MEA following the ejections of saline. However, due to the concentration gradient in the brain, the deflections were quickly recovered and a stable ACh signal baseline was attained.

The Effects of Neostigmine on ACh Efflux

Neostigmine has been shown to be a potent inhibitor of AChE, thus increasing extracellular levels of ACh. As a control, microdialysis collections taken during the course of the experiment were analyzed and graphed (Figure 9). The graph shows a dramatic increase in ACh concentrations after the onset of neostigmine (almost a 20 000% increase in ACh efflux from baseline). The analysis of the data shows a significant increase in ACh concentration

between the last baseline collection and the first collection after neostigmine administration. Thus it can be concluded that the neostigmine was working as predicted.

Interpretation of the Effects of Neostigmine on the Nicotine-Induced ACh Signal

The results also support the second hypothesis of this study. It was hypothesized that since the ACh signal was dependent upon the conversion of ACh by AChE, the administration of neostigmine, an AChE inhibitor, would markedly attenuate the nicotine-induced ACh signal peaks. The administration of neostigmine did significantly attenuate the peak amplitudes (a 71% decrease from pre-neostigmine amplitudes), as well as the clearance rates, for the ACh signal. This data supports the hypothesis that AChE is necessary for generating an ACh signal. This information is critical in understanding how the microelectrode array works. This experiment served to validate the importance of AChE, particularly endogenous AChE, in generating the ACh signal.

Neostigmine did not completely inhibit the all of the AChE present in the brain. This is evident from the generation of some signal, although markedly attenuated, even after the administration of neostigmine (Figure 6). Whether the uninhibited AChE was on the tips of the MEAs or from an endogenous source remains to be researched. However, due to the length of the perfusion period and the strength of the concentration of neostigmine used, it is unlikely that the uninhibited AChE was from an endogenous source in close proximity to the microdialysis probe.

Time Control Interpretation

The aim of the time control was twofold: to ensure that the neostigmine-induced peak and clearance attenuations were due to neostigmine and not to nicotinic receptor desensitization, and to control for the length of the experiment. The complete time course of an experiment was replicated, and aCSF was used in place of neostigmine. The analysis of the pre- and post- 90 minute peaks showed no significant differences. Thus, it can be concluded that while some nicotinic receptor desensitization may take place, the amount is not significant enough to detract from our attribution of attenuation to neostigmine inhibition of AChE.

It interesting to note that in Figure 6 there appears to be a linear regression in the top two traces which does not appear in the subtracted trace. It can be hypothesized that this linear regression, as it is eliminated when the traces are subtracted, is due to a factor other than ACh. Some possible explanations may include the reduction of the platinum recording sites, or a change in Ch levels over the course of the experiment. The source of the linear regression can be examined in future experiments.

The overall goal of this study was to provide further validation for the microelectrode array technique of measuring *in vivo* levels of neurotransmitters, specifically ACh in this study. The microelectrode arrays proved to be both sensitive and selective for ACh (see calibration in Figure 3), as well as able to take *in situ* readings that measured rapid ACh release and clearance. Finally, the method through which the ACh signal was generated was further examined by inhibiting the enzyme needed to initiate ACh breakdown. Therefore, this study was successful in providing more information about the microelectrode arrays.

General implications

The results of this study have further validated the MEA technology as an important development for the field of behavioral neuroscience. The temporal and spatial resolutions associated with this technique are critical in linking transient changes in neurotransmitter levels with changes in behavior. The ability to monitor ACh transmission *in situ* will give researchers the tools necessary to further explore neural circuitry, as well as neuropathologies.

The increased spatial and temporal resolutions are one of the key features of this technology. Microdialysis has done well in providing researchers with a vast amount of information about neural circuitry and neuronal transmission. The next step in this research is to harness the ability to look at how addictive drugs and disregulations in neural circuitry effect small populations of neurons in functionally distinct brain areas. The microelectrode array provides the resolutions needed to explore the brain on this level. The fine tuning of already known information will provide researchers with the ability to get to the cause of a problem. In research dealing with attention, the microelectrode array will be extremely valuable, as it will allow, for the first time, the ability to measure chemotransmission on the same time scale as behavioral events. For example, within our lab, the microelectrode array will eventually allow us to observe changes in neurotransmitters in areas such as the prefrontal cortex and nucleus accumbens as a rat performs a sustained attention task. By utilizing the microelectrode arrays in a variety of brain areas and specified for a variety of neurotransmitters, this technology will help us to develop a better understanding of all of the neural circuitry involved in attention. Additionally, in animal models of drug addiction, the microelectrode array will allow researchers

to explore the brain for irregularities in attentional processing, thus pointing scientists to the biological and psychological causes of craving and relapse associated with drug addiction.

This study utilized anesthetized rats as subjects, thus limiting the behavioral information gathered. In other currently ongoing studies, microelectrode arrays modified with headstages are being implanted in rats during survival surgeries. The result is being able to measure ACh transmission in awake, freely moving rats. This major development is the starting point for being able to record behavior and neurotransmitter release simultaneously. Stimuli such as novel odor and noise have been shown to evoke ACh release in awake, freely moving rats.

Additionally, work is currently underway to modify the microelectrode arrays themselves. Researchers are working to increase the number of channels available to record signals, as well as placing recording sites on both sides of the tips of microelectrode arrays. As the number of channels increases, so will the ability to monitor neurotransmitter release in different, distinct brain areas. This opportunity provides great promise for eventually understanding the phenomena of craving and relapse associated with drug addiction.

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FIGURE CAPTIONS

Figure 1.

A representation of the basal forebrain cortical cholinergic system and the mesolimbic dopaminergic system (from Sarter et al., 2005b). The diagram shows the complex relationship between both systems, as well as with other neural circuitry. These systems are critical in attentional processing and the phenomena of craving and relapse associated with drug addiction.

Figure 2.

The tip of the S-2 microelectrode array contains four platinum recording sites, arranged in two pairs. Each site measures 15 x 333 μm, with 100 μm separating the two pairs.

Figure 3.

The transduction scheme illustrates the generation of the ACh signal. Enzyme layers (AChE/ChO and ChO) are coated onto the microelectrode array and an exclusionary layer (m-PD) is electropolymerized onto the tip of the microelectrode array. These layers make the microelectrode array sensitive to and selective for ACh.

Figure 4

The calibration plot illustrates the sensitivity and selectivity of the microelectrode array. The top trace represents the combined ACh/Ch signal, the middle trace represents the Ch signal, and the bottom trace represents the subtracted, self-referenced ACh signal (nA). The ACh signal is responsive to step wise increases in ACh concentration, and is not affected by interferents. The abscissa is measured in seconds.

Figure 5.

Histological analyses were performed to ensure correct microelectrode array and probe placement. The figure on the top is a coronal section, and the figure on the bottom is a sagittal section. The coronal section shows the medial placement of the microelectrode array. The sagittal section illustrates both the microelectrode array (vertical placement) and microdialysis probe (30º angle) placements.

Figure 6.

Four nicotine ejections (1mM, 80 nL) resulted in ACh signals that quickly reached peak amplitude and were subsequently rapidly cleared to baseline. A 90 minute perfusion period of neostigmine (10 μM) significantly attenuated the ACh signal. The top trace is the combined ACh/Ch signal, the middle trace is the Ch signal, and the bottom trace is the subtracted, self referenced ACh signal (μM). The abscissa is measured in seconds.

Figure 7.

Pre- and post- neostigmine dependent variable data from the five subjects support the hypothesized attenuation of the ACh signal peaks and clearance times. Percent decrease of peak amplitude was also calculated.

Figure 8.

Pressure ejections (80 nL) of saline did not result in ACh signal (μM) peaks . Thus it can be concluded that the nicotine-induced ACh signal is not due to the pressure ejection of a solution into the brain. The abscissa is measured in seconds.

Figure 9.

The ACh microdialysis data show a significant increase in percent change of ACh efflux from baseline. The first collection represents the last baseline taken before neostigmine administration. Neostigmine perfusion increased levels of extracellular ACh by up to 20 000%.

Figure 10.

An analysis of the time control did not show any significant differences between pre- and post-aCSF perfusion ACh signal (μM) peaks. There was no attenuation of the ACh signal, thus it can be concluded that ACh signal attenuation is neither due to time nor nicotinic receptor desensitization. The top trace is the combined ACh/Ch signal, the middle trace is the Ch signal, and the bottom trace is the subtracted, self referenced ACh signal (μM). The abscissa is measured in seconds.

Figure 1

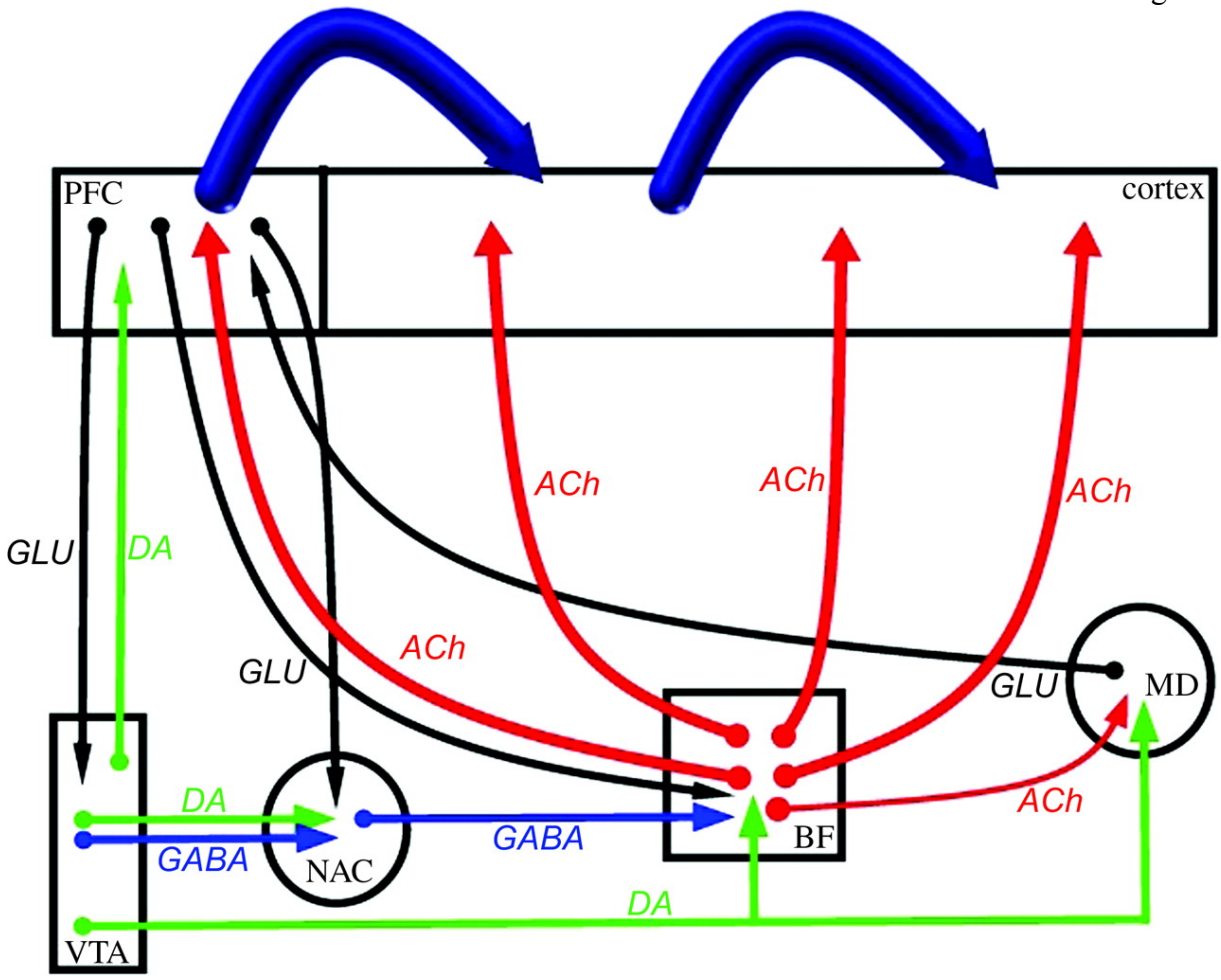
PFC
cortex
ACh
ACh
ACh
GLU
DA
ACh
GLU
GLU
MD
DA
GABA
NAC
GABA
BF
ACh
DA
VTA

Figure 2

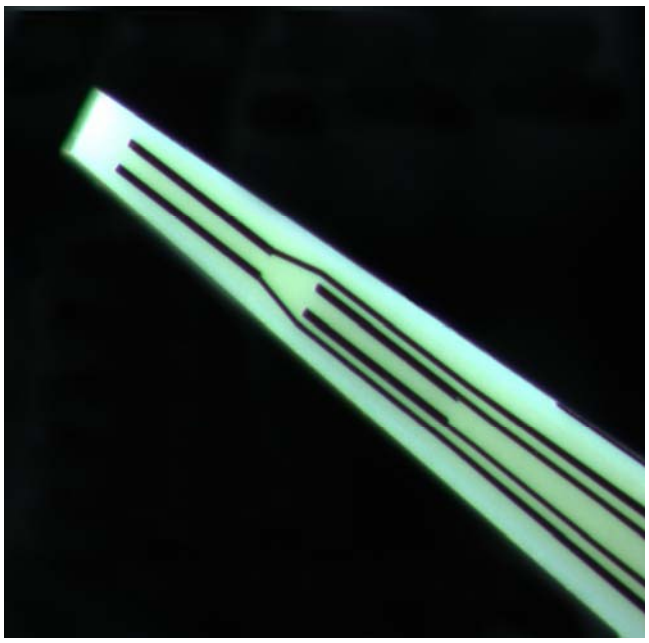

Figure 3

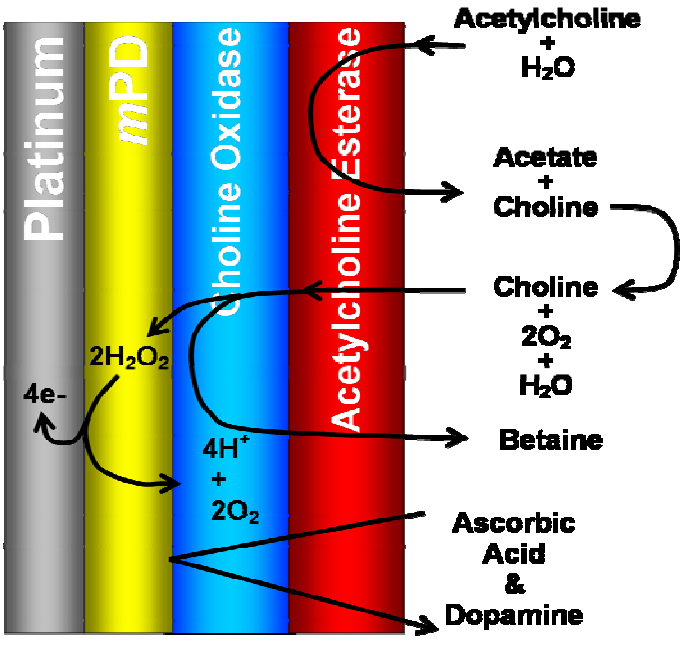
Platinum
mPD
Choline Oxidase
Acetylcholine Esterase
Acetylcholine
+
H2O
Acetate
+
Choline
Choline
+
2O2
+
H2O
Betaine
2H2O2
4e-
4H+
+
2O2
Ascorbic
Acid
&
Dopamine

Figure 4

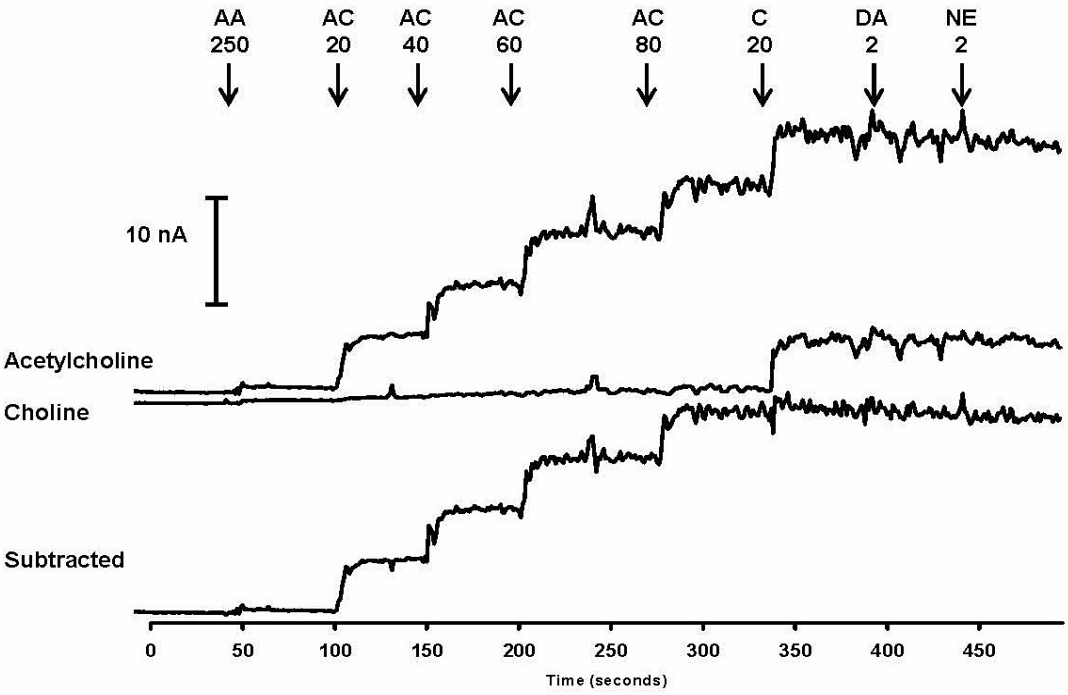
AA
250
AC
20
AC
40
AC
60
AC
80
C
20
DA
2
NE
2
10 nA
Acetylcholine
Choline
Subtracted
0
50
100
150
200
250
300
350
400
450
Time (seconds)

Figure 5

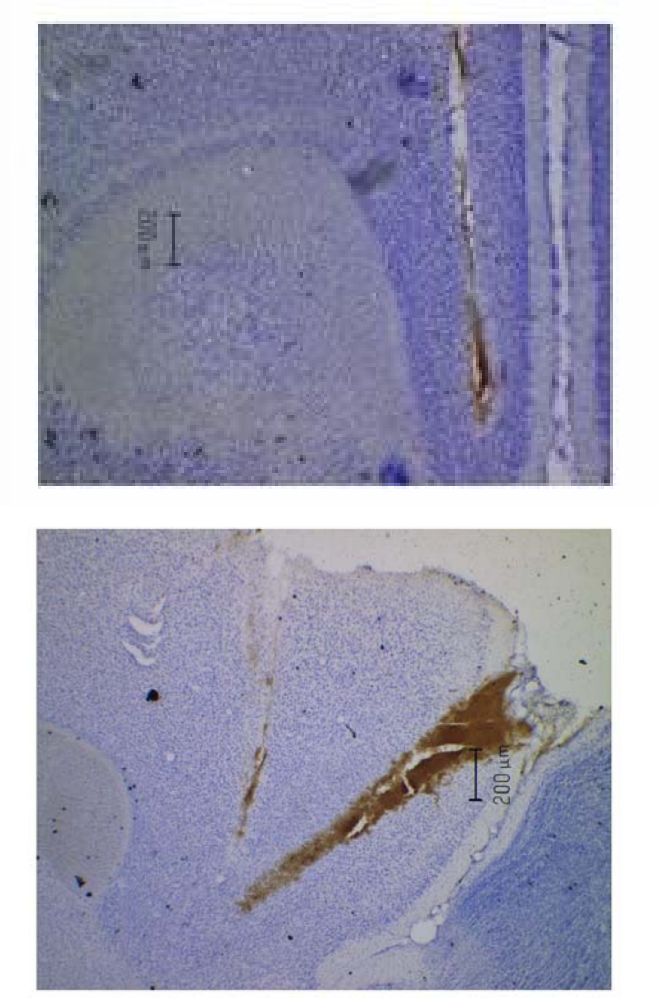

Figure 6

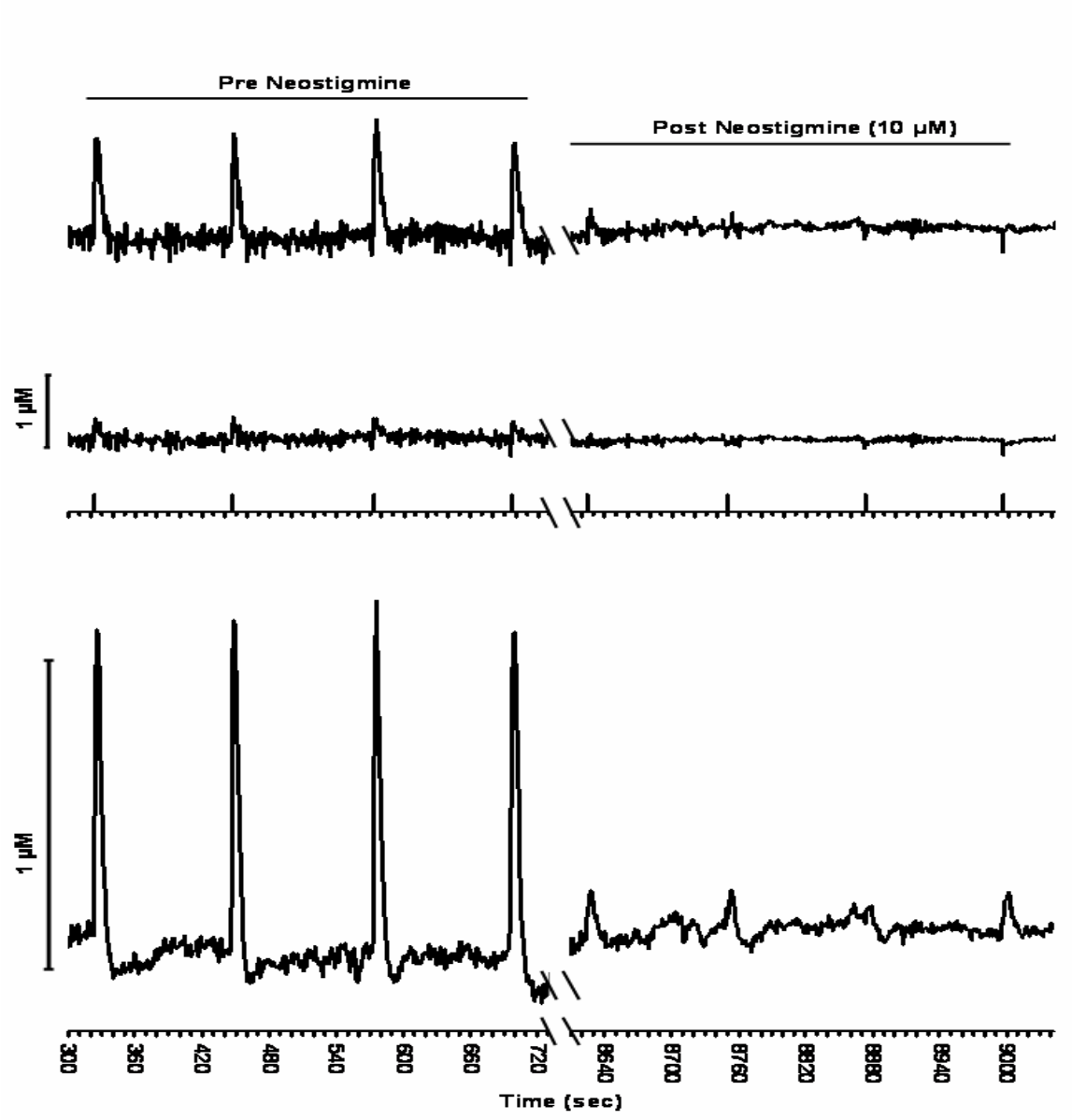
Pre Neostigmine
Post Neostigmine (10 µM)
1 µM
1 µM
300
360
420
480
540
600
660
720
8640
8700
8760
8820
8880
8940
9000
Time (sec)

Figure 7

Subjects	Amp. (μM)		% Decrease of Amp.	Clearance (μM/sec)		T80 (sec)	
	Pre Neostigmine	Post Neostigmine		Pre Neostigmine	Post Neostigmine	Pre Neostigmine	Post Neostigmine
1	2.60	1.60	38.67	0.14	0.01	3	17
2	0.77	0.04	94.33	0.13	8.61×10^{-5}	3	1
3	3.22	1.67	48.01	0.40	0.15	9	8
4	1.03	0.14	86.83	0.20	0.01	10	12
5	10.30	1.00	90.27	7.17	1.09	3	3
Average	**3.58**	**0.89**	**71.62**	**1.61**	**0.25**	**6**	**8**

Figure 8

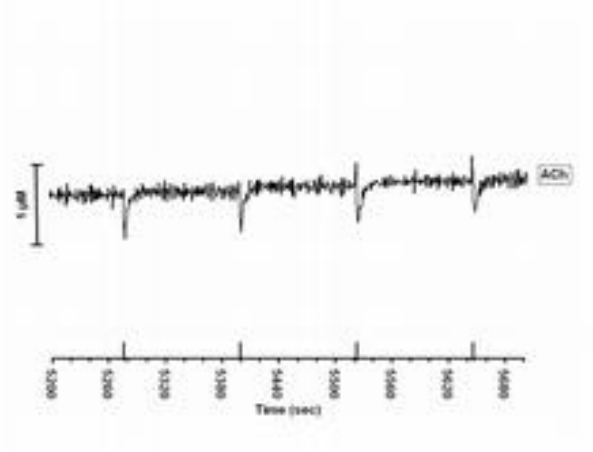

Figure 9

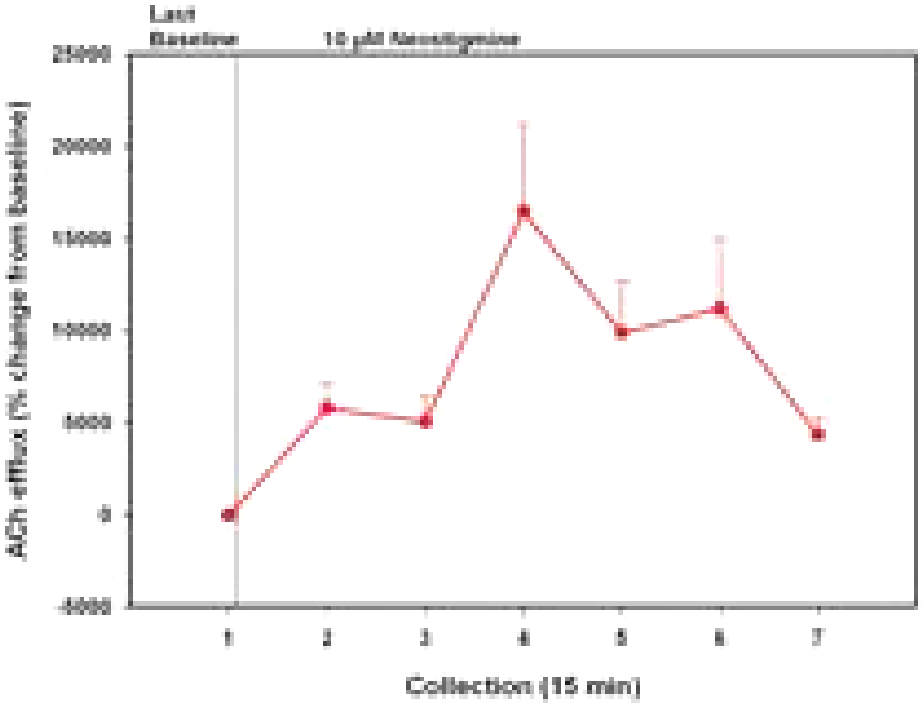
Last
Baseline
10 µM Neostigmine
ACh efflux (% change from baseline)
25000
20000
15000
10000
5000
0
-5000
1
2
3
4
5
6
7
Collection (15 min)

Figure 10